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- P. Pro; Q. Clin; R. Arg; S. Ser; T. Thr; V. Val; W. Trp; and Y. Tyr. X indicates any residue.
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 20. Preparation of nondetergent cell lysate was carried out using buffer conditions similar to procedures described in (30) except no detergent was included. Gel filtration chromatography was done according to the published protocol in X.-D. Li *et al.* [*J. Biol. Chem.* **272**, 705 (1997)].
 21. Phosphorylation in vitro: COS 7 cells were transfected with mammalian expression plasmids, changed with phosphate-free media after 24 hours, and harvested after 48 hours. The cells were lysed in Hepes-buffered saline (140 mM NaCl, 2.7 mM KCl, and 15 mM Hepes), pH 7.4 and 1% Triton X-100 and centrifuged at 10,000g for 5 min. The extract was added to 10 μ l of protein G-agarose beads with cross-linked anti-HA antibody. The beads were collected and washed three times with Hepes-buffered saline. The pellet was then resuspended in 20 μ l kinase buffer (35 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.5 mM EGTA, 0.1 mM CaCl₂, and 1 mM phenylphosphate). After supplementing the reaction with the indicated amounts of GST ZIPs or control protein, phosphorylation was activated by adding 5 μ l of phosphatidylserine (1 mg/ml) and 0.5 μ l of [³²P]- γ -ATP (10 mCi/ml). Unless specified, all phosphorylation reactions were incubated for 30 min at 30°C. The reaction was stopped by addition of SDS loading buffer. After boiling for 5 min, the samples were separated by SDS-PAGE. Protein phosphorylation was detected by autoradiography using Kodak scientific imaging films. Quantification of the relative amount of radioactivity was performed with a phosphor imager (FUJIX BAS 1000, Fuji, Tokyo).
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 26. Antibodies for ZIP and Kv β 2 were generated by immunizing rabbits with GST-fusion proteins corresponding to the full-length cDNAs of rat ZIP1 and Kv β 2. The PKC ζ and PKC β antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).
 27. The HA-tagged Kv β 2 was expressed by transient transfection and cell lysates from HA-Kv β 2 and mock-transfected cells were prepared according to the protocol described in Yu *et al.* (10). Affi-gel 10 (Bio-Rad, CA) was used to conjugate GST control, GST-ZIP1 and GST-ZIP2. The binding reactions were performed using 20 μ l beads and 100 μ l of cell extracts in a buffer containing 20 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol. After incubation on ice for 1 hour, the beads were washed three times, and the bound material was released by boiling the beads in SDS-sample buffer. The Kv β 2 binding was tested by fractionating the bound material on SDS gel followed by immunoblot with anti-HA antibody.
 28. In situ hybridization was performed using a DIG-RNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Briefly, DNA templates for riboprobe synthesis were prepared by digesting plasmid clones containing the full-length rat Kv β 2, ZIP1, and PKC ζ cDNAs. After linearization, DIG-labeled RNA probes were prepared by in vitro transcription. Adult Sprague-Dawley rats were used for analysis of Kv β 2, ZIP1/2 and PKC ζ mRNA expression. Animals were anesthetized and decapitated, and their brains were removed immediately and fixed in 4% paraformaldehyde for 2 hours. Frozen sections (12 μ m) were cut on a cryostat, thaw mounted onto SuperfrostPlus slides, and air-dried. All solutions were prepared in deionized H₂O treated with 0.1% (V/V) diethylpyrocarbonate and autoclaved. Sections were fixed by immersion in 4% paraformaldehyde in PBS, pH 7.4, then briefly rinsed twice with PBS. After treatment with Proteinase K, sections were refixed in 4% paraformaldehyde. The sections then were acetylated by immersion in 0.1 M triethanolamine containing 0.25% acetic anhydride, permeabilized by 1% Triton X-100, and rinsed twice with PBS. Prehybridization was

carried out at 4°C overnight with prehybridization solution (50% formamide, 5 \times SSC, 5 \times Denhardt's solution, 250 μ g/ml yeast tRNA, and 500 μ g/ml salmon sperm DNA). For hybridization, the sections on each slide were covered by a prehybridization solution containing 1 μ g/ml of cRNA probe, incubated at 65°C overnight in a humid chamber. Sections were immersed sequentially in 0.2 \times SSC twice and buffer 1 (0.1 M Tris pH 7.5, 0.15 M NaCl) twice. The sections were covered by 1:2000 anti-Digoxin antibody in buffer 2 (1% inactivated normal goat serum in buffer 1) and incubated at 4°C overnight. After rinsing with buffer 1 and buffer 3 (0.1 M Tris pH 9.5, 0.1 M NaCl, and 50 mM MgCl₂), the sections were developed with a solution containing 1.2 mg levamisole and 300 μ l NBT/BCIP in 5 ml development buffer containing 0.1 M Tris (pH 9.5), 0.1 M NaCl, and 50 mM MgCl₂.

29. J. Gong *et al.*, unpublished data.
30. Immunoprecipitation experiments were performed using cerebellum extracts from adult rat brain. The extracts were prepared by homogenizing rat cerebellum in a lysis buffer containing 10 mM Tris HCl (pH 8.0), 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 10 mM Na₂ and protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 1 μ g/ml pepstatin). Homogenization was carried out at 4°C using a Dounce homogenizer with a typical tissue to buffer ratio of 8 ml buffer per gram of tissue. After 20 strokes of homogenization, homogenates were centrifuged at 10,000g for 5 min and the supernatants were collected. To conjugate antibodies to Protein A-agarose, 40 μ l of Protein A beads (Sigma Chemical, St. Louis, MO) in 1 ml of PBS were first incubated overnight with 20 μ l of antiserum at 4°C. After removal of unbound material, the agarose was washed three times with PBS, resuspended in 500 μ l of 0.2 M sodium borate (pH 9.0). The cross-linking was initiated by adding dimethylolmelitimide to a final concentration of 20 mM.

After 30 min incubation at room temperature, the reaction was stopped by addition of 500 μ l of 0.2 M ethanolamine. After 2 hours incubation, the conjugated protein A-agarose was washed three times with PBS. The antibody binding was carried out by incubating 200 μ l of soluble lysate with 10 μ l of antibody-protein A agarose. After overnight incubation at 4°C, the Protein A agarose was collected by centrifugation at 5,000g for 2 min, and washed three times with PBS. The individual Protein A pellets were treated with 10 μ l of 2 \times SDS sample buffer at 100°C for 5 min. The soluble protein samples were separated by SDS-PAGE. The immunoprecipitated polypeptides were detected by immunoblot using the corresponding antibodies as indicated.

31. The PC12 culture and NGF stimulation were carried out essentially as described (24). Briefly, 10⁵ cells were seeded in a 3.5-cm dish and allowed to grow for 24 hours in the presence of 10% fetal bovine serum and 5% heat inactivated horse serum. The NGF stimulation was initiated by adding purified NGF to a final concentration of 0.1 μ g per ml. The cells were allowed to grow for 72 hours and harvested for analyses.
32. We thank D. Ginty for providing purified NGF, A. Lanahan and P. Worley for cDNA libraries, J. Baraban for anti-translin antibody, Y. Ono for rat PKC ζ cDNA, L. Roman for in situ hybridization technique, M. Regan for RT-PCR technique, S. Wang for the control GST fusion protein and R. Butzner for help with manuscript preparation. We also thank C. Montell, P. Gillespie, and members of the Li Lab for comments on this manuscript. M.B. was a NSF predoctoral fellow. R.H. is supported in part by a postdoctoral fellowship from American Heart Association. M.L. is supported by a grant from NIH (NS33324) and an American Heart Association-Pfizer award.

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In Vivo Protein Transduction: Delivery of a Biologically Active Protein into the Mouse

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Delivery of therapeutic proteins into tissues and across the blood-brain barrier is severely limited by the size and biochemical properties of the proteins. Here it is shown that intraperitoneal injection of the 120-kilodalton β -galactosidase protein, fused to the protein transduction domain from the human immunodeficiency virus TAT protein, results in delivery of the biologically active fusion protein to all tissues in mice, including the brain. These results open new possibilities for direct delivery of proteins into patients in the context of protein therapy, as well as for epigenetic experimentation with model organisms.

Currently, efficient delivery of therapeutic compounds, peptidyl mimetics, and proteins into cells in vivo can be achieved only when the molecules are small—typically less than 600 daltons (1). Delivery of bioactive peptides across the blood-brain barrier, for ex-

ample, is generally restricted to small (six amino acids or less), highly lipophilic peptides (1). Gene therapy (2) is one promising method for circumventing this problem, but conditions for high-efficiency targeting and long-term protein expression have yet to be discovered.

We have focused on an alternative approach of “protein transduction,” or protein therapy, to address this problem. In this method (3), full-length fusion proteins are generated that contain an NH₂-terminal 11 amino acid

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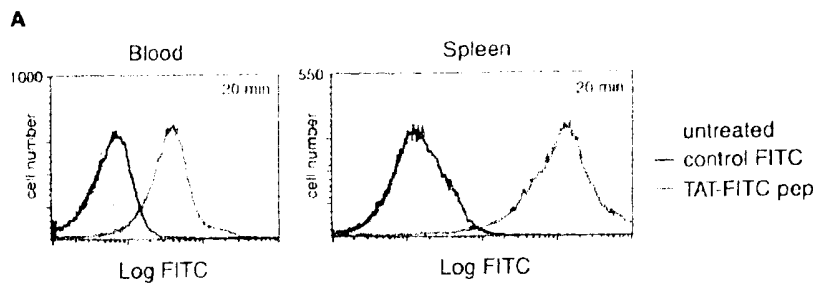


Fig. 1. Transduction of TAT-FITC peptide into mice. **(A)** Flow cytometry of whole blood cells (left) and splenocytes (right) isolated from mice 20 min after ip injection of TAT-FITC peptide or control free FITC. **(B)** Fluorescence confocal microscopy of hemispheric sagittal brain sections (top) and skeletal muscle tissue sections (bottom) isolated from mice 20 min after ip injection as in **(A)**. Scale bars, 100 μ m.

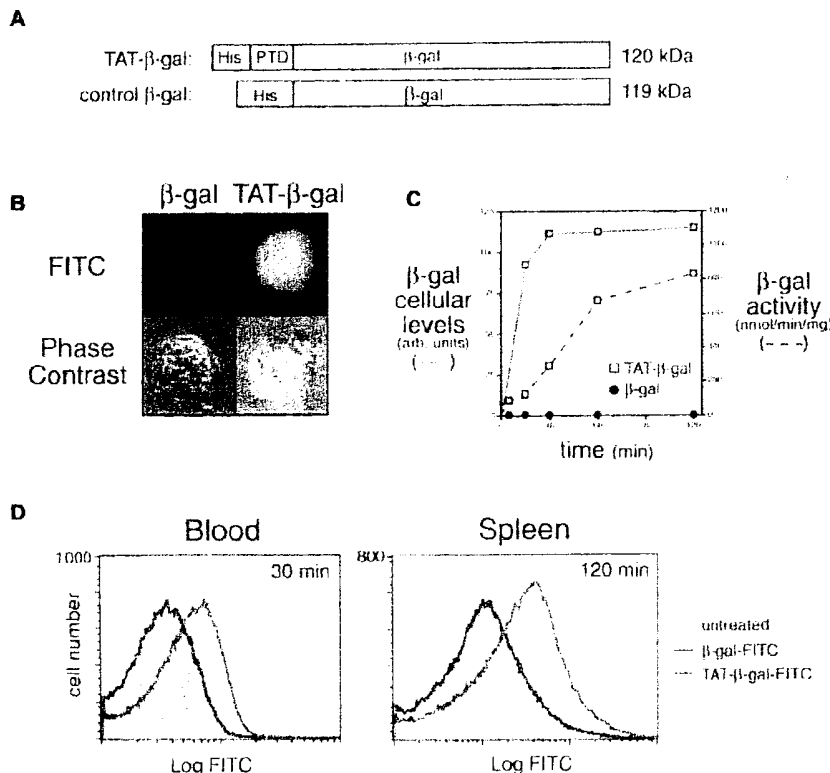
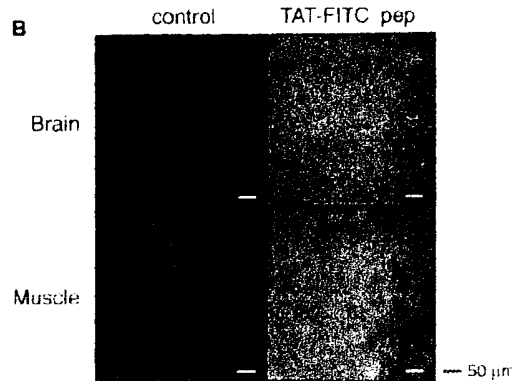


Fig. 2. Transduction of TAT- β -Gal into cultured cells. **(A)** Diagram of TAT- β -Gal and control β -Gal fusions. **(B)** Fluorescence (top) and light (bottom) confocal microscopy showing that TAT- β -Gal-FITC entered Jurkat T cells, whereas β -Gal-FITC did not. **(C)** Concentration (left axis) and enzymatic activity (right axis) of HepG2 cells treated with TAT- β -Gal (open squares) or β -Gal (solid circles). β -Gal concentration was determined by immunoblot analysis. **(D)** Flow cytometry of whole blood cells (left) and splenocytes (right) performed at the indicated times after ip injection of mice with TAT- β -Gal-FITC or control β -Gal-FITC.

protein transduction domain (PTD) from the human immunodeficiency virus (HIV) TAT protein [first identified in 1988 (4)]. These proteins are then purified under denaturing conditions (3). Protein transduction occurs in a rapid, concentration-dependent fashion that appears to be independent of receptors and transporters (5) and instead is thought to target the lipid bilayer component of the cell membrane. Thus, in principle, all mammalian cell types should be susceptible to protein transduction, and indeed we have used this technology to transduce over 50 proteins ranging in size from 15 to 120 kD into a wide variety of human and murine cell types in vitro (3, 6).

To determine whether this method could be used to deliver peptides in vivo, we synthesized a 15-oligomer peptide containing the 11 amino acid TAT PTD, preceded by an NH₂-terminal fluorescein isothiocyanate (FITC)-Gly-Gly-Gly-Gly motif that rapidly transduced into ~100% of cultured cells (7). We next injected C57BL/6 mice intraperitoneally with 1.7 nmol of the TAT-FITC peptide or with control free FITC and monitored the appearance of fluorescent cells (8). Flow-activated cell sorting (FACS) analysis of whole blood isolated 20 min after intraperitoneal (ip) injection with TAT-FITC peptide revealed a strong fluorescence signal in ~100% of blood cells (Fig. 1A, left). Blood cells from mice injected with control free FITC showed a small constant increase in background fluorescence (9) that was likely due to uptake of FITC from the peritoneum by the lymphatic system. We also analyzed splenic cells by performing a splenectomy 20 min after ip injection of the mice. FACS analysis revealed transduction of TAT-FITC peptide into all splenic cells, including T cells, B cells, and macrophages (Fig. 1A, right). Control ip injections of equimolar amounts of free FITC showed only a minor increase in fluorescence above background levels. Thus, the injected TAT PTD peptide was rapidly transduced into all blood and splenic cells.

We next studied the uptake of the TAT peptide into brain tissue and skeletal muscle. Tissues were dissected from mice 20 min after ip injection with TAT-FITC peptide (8), and cryostat sections were prepared. Fluorescence confocal microscopy analysis of 10- μ m hemispheric sagittal brain sections revealed a strong signal in all areas of the brain from TAT-FITC peptide-injected mice, whereas the signal in control FITC-injected mice remained at background levels (Fig. 1B, top). Fluorescence photobleaching was observed when TAT-FITC peptide sections were subjected to prolonged excitation, providing further evidence that the TAT-FITC peptide was in

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the brain section. Skeletal (quadriceps) muscle also showed a significant fluorescence signal from TAT-FITC-injected mice as compared to control FITC-injected mice (Fig. 1B, bottom).

To determine whether a large biologically active protein could be successfully transduced *in vivo*, we used the 116-kD β -galactosidase (β -Gal) protein (10). An NH₂-terminal TAT- β -Gal fusion protein (120 kD) was generated (11), as was a control β -Gal fusion protein missing the 11-amino acid TAT PTD but retaining the rest of the NH₂-terminal leader (119 kD) (Fig. 2A). Fluorescence confocal microscopy of cultured cells treated with FITC-labeled TAT- β -Gal (12) revealed that the protein was inside the cells, whereas control β -Gal-FITC was not detectable, and control free FITC was bound to the cellular membrane (Fig. 2B). Immunoblot analysis revealed that the TAT- β -Gal was rapidly transduced into cultured cells, reaching near maximum intracellular concentrations in less than 15 min, whereas the control β -Gal protein did not transduce into cells even after 2 hours (Fig. 2C). Unexpectedly, the enzymatic activity of TAT- β -Gal peaked about 2 hours later than did the intracellular concentration (13) (Fig. 2C). This lag may reflect a slow posttransduction refolding rate of the protein by intracellular chaperones such as HSP90 (14).

We next intraperitoneally injected FITC-labeled TAT- β -Gal and control β -Gal proteins into mice. Flow cytometry of blood and splenic cells isolated 30 and 120 min, respectively, after ip injection demonstrated the presence of TAT- β -Gal-FITC in all blood and splenic cells (Fig. 2D). In contrast, the control β -Gal-FITC was not detectable (Fig. 2D). Similar results were obtained in experiments with two other FITC-labeled TAT fusion proteins: TAT-Cdk2-DN [human Cdk2 (cyclin-dependent kinase-2) dominant negative] (36 kD) (3) and TAT-CAK1 [yeast CAK1 (Cdk-activating kinase-1)] (47 kD) (15, 16).

The mice were then analyzed for β -Gal enzyme activity. Tissue samples from the liver, kidney, heart muscle, lung, and spleen were isolated at 4 and 8 hours after ip injection, sectioned (in 10- and 50- μ m sections), and assayed by X-Gal staining (8) (Fig. 3). Liver, kidney, and lung tissues from TAT- β -Gal-injected mice showed strong and uniform β -Gal activity across the tissue sections at 4 and 8 hours after ip injection. The heart samples also showed strong β -Gal activity throughout the muscle fibers. Sections from control β -Gal-injected mice showed either no staining or sporadic weak staining that was likely due to lymphatic uptake of control β -Gal from the peritoneum. The control β -Gal-treated

kidney showed weak staining, presumably reflecting clearance from the bloodstream. The spleen showed strong β -Gal activity in the red pulp areas and much weaker activity in the white pulp areas (Fig. 3), which are principally composed of T and B cells. This is at odds with the FACS transduction analysis (Fig. 2D); however, T and B cells are known to contain a β -Gal inhibitory activity (17).

To determine whether the protein crossed the blood-brain barrier, we performed X-Gal staining on mid-hemispheric sagittal brain sections from mice at various times after injection of TAT- β -Gal or control β -Gal (Fig. 4). Brain sections from mice analyzed 2 hours after ip injection with TAT- β -Gal showed strong activity that was principally localized around blood vessels, with minimal activity being present in the surrounding parenchyma (Fig. 4A). However, by 4 hours after ip injection, all regions of the brain showed strong β -Gal activity. In contrast, mice injected with control β -Gal showed no β -Gal activity in the brain at 2 hours (15), 4 hours (Fig. 4A), or 8 hours

after injection (15). By 8 hours after ip injection of TAT- β -Gal, enzyme activity resided primarily in cell bodies throughout all regions of the brain, and to a lesser extent in the surrounding white matter. Because cell bodies are mainly composed of nuclei, this raises the possibility that the protein enters the nucleus, perhaps via the embedded nuclear localization signal in the TAT PTD (18) (Fig. 4A). The blood-brain barrier remained intact in TAT- β -Gal-treated mice, as measured by the absence of extravasated coinjected Evan's blue albumin complexes in brain sections (19) (Fig. 4B). In addition, a low-magnification coronal brain section revealed β -Gal activity in cell bodies throughout the brain of TAT- β -Gal-treated mice 8 hours after injection (Fig. 4C).

A previous attempt to transduce β -Gal chemically cross-linked to the TAT PTD into mice resulted in sporadic and weak β -Gal activity in a limited number of tissues, with no activity detected in the kidney or brain (20). The increased transduction potential reported here likely reflects the in-frame fusion

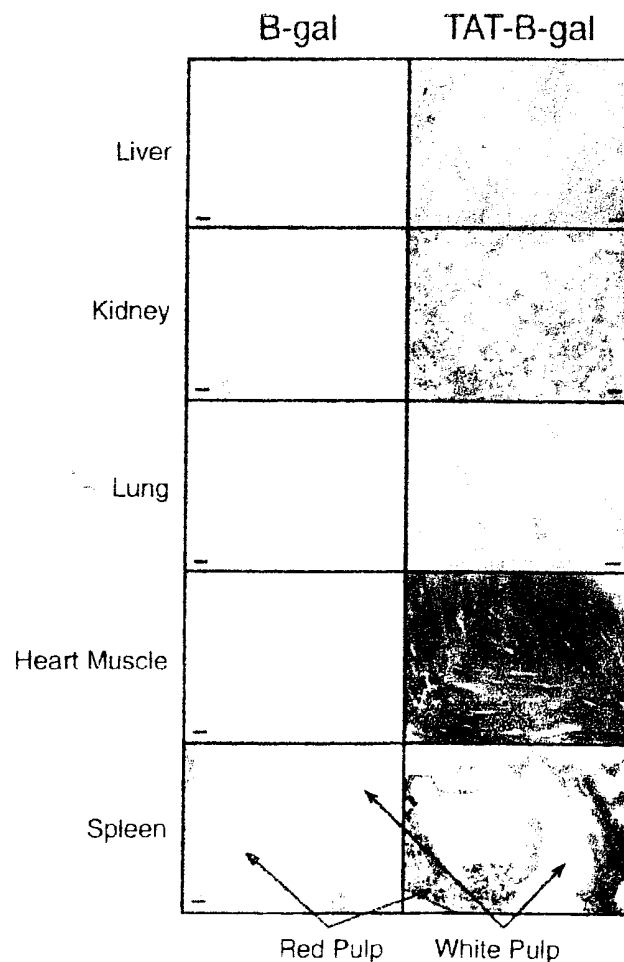
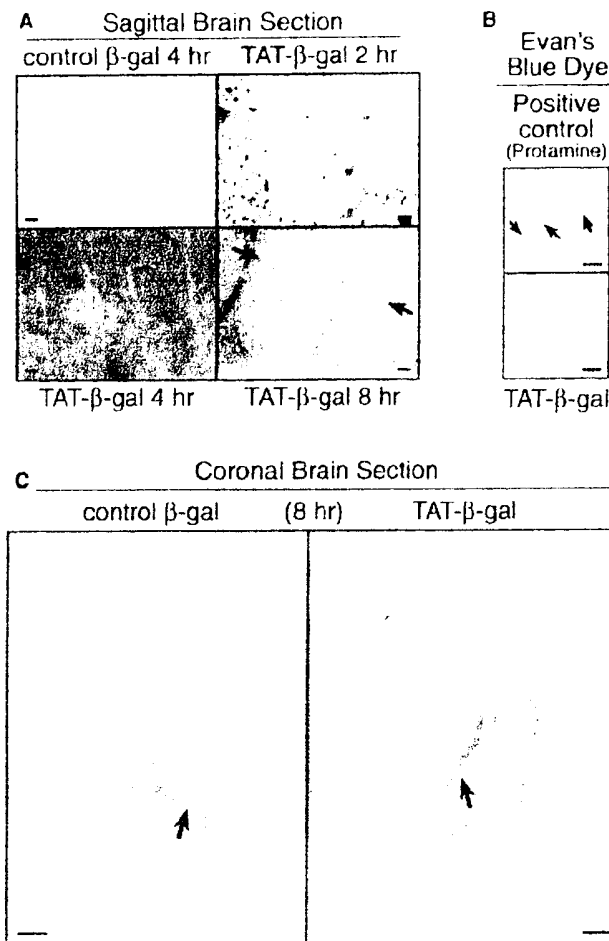


Fig. 3. Transduction of TAT- β -Gal into mice. Analysis of β -Gal enzymatic activity assessed by X-Gal staining in the liver, kidney, lung, heart muscle, and spleen is shown. Samples were analyzed 4 hours after ip injection of proteins, except for kidney tissue, which was analyzed 8 hours after injection. There is weak β -Gal activity in the white pulp areas of the spleen. Sections were developed for 4 hours. Scale bars, 100 μ m.

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Fig. 4. Transduction of TAT- β -Gal across the blood-brain barrier. (A) β -Gal activity (X-Gal staining) in hemispheric sagittal brain sections from mice injected intraperitoneally with TAT- β -Gal or control β -Gal; sections were made after the indicated times. Eight hours after ip injection, TAT- β -Gal localized primarily to the nuclei of cell bodies (arrows) throughout the brain section. Sections were developed in X-Gal for 16 hours. Scale bars, 100 μ m. (B) Extravasated Evan's blue dye was detected adjacent to blood vessels throughout the brain sections (50 μ m) from mice treated with the protamine positive control (top) but was not detected in sections from TAT- β -Gal-treated mice (bottom). Scale bars, 100 μ m. (C) β -Gal activity in a low-magnification coronal brain section from mice 8 hours after ip injection with control β -Gal (left) or TAT- β -Gal (right), showing activity throughout the brain. Arrows indicate the region of endogenous β -Gal activity. Scale bars, 500 μ m.



and purification strategy (3). Furthermore, we have engineered a series of artificial PTDs that show dramatically enhanced transduction potential in cultured cells (21).

The transduction of peptides and proteins into mice lays the groundwork for future epigenetic complementation experiments in model organisms and for the eventual transduction of therapeutic proteins into patients in the form of protein therapy. Therapy with biologically active full-length proteins will allow access to the built-in evolutionary specificity of these proteins for their targets, thereby potentially avoiding the nonspecific effects sometimes seen with small molecule therapies. To this end, we have described an experimental anti-HIV protein therapy based on a transducible HIV protease-activated caspase-3 that selectively induces apoptosis in HIV-infected cells (22). Finally, this methodology opens new possibilities for the development of vaccines and protein therapies for cancer and infectious diseases. Potential immune responses and toxicity associated with long-term transduction of proteins in

vivo are important issues that remain to be examined. Along these lines, we note that injection of a mouse with 1 mg of a TAT PTD fusion protein per kilogram of body weight each day for 14 consecutive days produced no signs of gross neurological problems or systemic distress (23).

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- Synthetic TAT peptides contained an NH_2 -terminal FITC-Gly residue that resulted in identical coupling rates between peptides [FITC-G-GGG-YGRKKRRQ-RRR (G, Gly; K, Lys; Q, Gln; R, Arg; Y, Tyr)]. All peptides were resuspended in water, and concentrations were normalized by fluorescence values from a fluorometer. Transduction into cultured Jurkat T cells was measured by FACS (Becton Dick-

inson) as performed in (3) and by fluorescence confocal microscopy in 4% paraformaldehyde-fixed cells as performed in (3). Control FITC was found to be associated with cells only on the outside of the cell membrane (15). Human Jurkat T cells and HepG2 hepatocellular carcinoma cells were maintained as in (3, 6).

- Sixty-five 4- to 8-week-old C57BL/6 mice were injected intraperitoneally with 1.7 nmol of TAT-FITC peptide or with control FITC in 500 μ l of phosphate-buffered saline (PBS) or 100 to 500 μ g of TAT- β -Gal or β -Gal control protein in 0.5 to 2.0 ml of PBS and 10% glycerol. Whole blood cells were isolated from the orbital artery, and splenocytes were isolated at indicated time points and analyzed by FACS. Treated mice were killed, and tissues were harvested and frozen in Histo Prep media (Fisher Scientific). Sections (10 to 50 μ m) were cut on a cryostat, fixed in 0.25% glutaraldehyde for 15 min, and developed for 4 hours (liver, kidney, lung, heart, and spleen) or 16 hours (brain) in 0.2% X-Gal solution (10) or analyzed by fluorescence confocal microscopy. All mice injected with either TAT-FITC peptide or TAT- β -Gal yielded positive results. All animal procedures were performed in accordance with institutional guidelines.
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- TAT- β -Gal and β -Gal were labeled with FITC as in (3).
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